The Coumarin Glucoside, Esculin, Reveals Rapid Changes in Phloem-Transport Velocity in Response to Environmental Cues

Short title:

Esculin, a sucrose proxy for phloem transport

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The coumarin glucoside, esculin, reveals rapid changes in phloem-transport velocity in response to environmental cues

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Summary:

Esculin, used as a sucrose mimic, shows that the velocity of phloem transport is regulated by environmental cues, changes in sucrose levels, and the expression of the sucrose transporter *AtSUC2*.

List of author contributions:

KK conceived, designed and performed the experiments; AP and ST performed preliminary experiments; KK and KO wrote the paper.

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Abstract

The study of phloem transport and its vital roles in long distance communication and carbon allocation have been hampered by a lack of suitable tools that allow high-throughput, real-time studies. Esculin, a fluorescent coumarin glucoside, is recognised by sucrose transporters, including AtSUC2, which loads it into the phloem for translocation to sink tissues. These properties make it an ideal tool for use in live-imaging experiments where it acts as a surrogate for sucrose. Here we show that esculin is translocated with a similar efficiency to sucrose and, because of its ease of application and detection, demonstrate that it is an ideal tool for in vivo studies of phloem transport. We used esculin to determine the effect of different environmental cues on the velocity of phloem transport. We provide evidence that fluctuations in cotyledon sucrose levels influence phloem velocity rapidly, supporting the pressure-flow model of phloem transport. Under acute changes in light levels the phloem velocity mirrored changes in the expression of *AtSUC2*. This observation suggests that under certain environmental conditions, transcriptional regulation may affect the abundance of AtSUC2, and thus regulate the phloem transport velocity.

Introduction

The phloem of higher plants consists of a highly developed network of specialized enucleate cells known as sieve elements (SEs), connected to their adjacent metabolically-supportive companion cells (CCs) by specialized plasmodesmata called Pore Plasmodesmata Units (PPUs; van Bel, 1996; Oparka and Turgeon, 1999; Heo et al., 2014). Sieve elements are connected end to end by perforated sieve plates, allowing long-distance translocation of photosynthetically derived assimilates and a wide-range of solutes, hormones, proteins and RNAs (Wardlaw, 1990; Molnar et al., 2010; Bishopp et al., 2011; Liu et al., 2012; Paultre et al., 2016). The phloem network of plants thus performs key roles in carbon allocation and in the long-distance movement of systemic macromolecules.

The flow of the sucrose-rich sap in the phloem is thought to occur by mass flow, as originally envisaged by Münch (1930). Sugars, such as sucrose, are loaded into the SEs of the phloem in photosynthetically active tissues (sources). The high concentration of sucrose in these phloem cells osmotically attracts water from the xylem, increasing the hydrostatic pressure within SEs and driving flow from source to sink regions of the plant where the sucrose is unloaded and used in metabolism and growth. The removal of solutes and water from the sites of phloem unloading maintains an osmotic gradient along the SE files (sieve tubes) and creates the pressure differential required to drive long-distance flow (Knoblauch et al., 2016; Ross-Elliott et al., 2017).

Sucrose can be loaded into the phloem either symplastically or apoplastically. In symplastic loading, sucrose reaches the companion cells through multiple plasmodesmata that connect them with the surrounding bundle sheath and parenchyma cells. It is either transported into the phloem by simple passive diffusion (diffusive loading), or converted into high-molecular weight polymers such as stachyose and raffinose (polymer-trapping), with subsequent movement through the large-diameter PPUs between CC and SE; (van Bel, 1996; Rennie & Turgeon, 2009). Apoplastic loaders, such as Arabidopsis (*Arabidopsis thaliana*), use active proton-mediated transport via SUCROSE TRANSPORTERS (SUTs) to load sucrose into the CC from the apoplast against a concentration gradient (Sauer, 2007; Rennie & Turgeon, 2009). There are a number of SUTs and other sugar transporters present in Arabidopsis, but *SUCROSE TRANSPORTER2* (*AtSUC2*) is expressed specifically in CCs and is responsible

for sucrose loading into the collection phloem in the minor veins of leaves (Truernit & Sauer, 1995).

Recently, the simplicity of the Münch pressure-flow hypothesis has been questioned, one argument being that the magnitude of hydrostatic pressure gradients in large trees may be too low to drive the observed rates of flow (Turgeon, 2010). However, newer experimental methods, incorporating mathematical modelling, have provided data in support of the original Münch model (Jensen et al., 2011; Knoblauch et al., 2016).

Despite the fundamental importance of the phloem in assimilate distribution, basic questions remain as to how phloem transport responds to environmental changes. Indeed, considering the extensive literature on carbon partitioning in plants, there have been very few studies in which phloem transport velocity has been measured *in planta*. Since the 1970s, several studies have used ^{14}C or ^{11}C isotopes to measure rates of phloem transport in large plants. This was usually achieved by placing two or more Geiger-Muller (GM) tubes along the phloem pathway to track the movement of the radioactive solute front or by freeze-drying and exposing the tissue to autoradiographs (Christy and Fisher, 1978; Madore and Lucas, 1987; Minchin and Thorpe, 2003). However, these studies had limited resolution and were not suitable for use on very small plants or seedlings, such as Arabidopsis. More recently, phloem transport has been investigated using Magnetic Resonance Imaging (MRI) techniques or refining the use of radioactive tracers such as ^{11}C, for example, with the use of specialised hydroponic root chambers (Köckenburger et al., 1997; Peuke et al., 2001; Windt et al., 2006; Mullendore et al., 2010; Gould et al., 2012). However, these methods are expensive, time-consuming, and lack the resolution needed to study phloem transport at the level of the SE (Gould et al., 2012; Ohmae, et al., 2013; Kölling et al., 2015). Fluorescent tracers, such as carboxyfluorescein diacetate (CFDA), which translocate in the phloem, were first described over twenty years ago (Grignon et al., 1989), but they have only rarely been used to measure the velocity of phloem transport and are more often used to confirm that phloem transport has simply occurred (Oparka et al., 1994; Wright and Oparka, 1996; Jensen et al., 2011; Savage et al., 2013).

We recently described a range of fluorescent probes that are translocated in the phloem of Arabidopsis and which allow *in-planta* analysis of phloem transport (Knoblauch et al., 2015; Ross-Elliott et al., 2017). One of these, the coumarin glucoside esculin, is loaded into the Arabidopsis phloem specifically by the AtSUC2 transporter, and does not enter the phloem in

atsuc2 knockout seedlings, making it a potential surrogate for sucrose in phloem transport studies (Reinders et al., 2012; Knoblauch et al., 2015; De Moliner et al., 2018). Here we describe the use of esculin to measure the phloem transport velocity (PTV) in response to differing environmental conditions. We show that PTV can be measured rapidly in intact seedlings in a high-throughput manner. Our results provide evidence that fluctuations in leaf sucrose levels may influence PTV, supporting the pressure-flow model of phloem transport. Our data also show that under acute changes in the light environment, the rate of phloem transport mirrors the transcriptional level of *AtSUC2* in leaves, suggesting that AtSUC2 expression in CCs may regulate the PTV under certain environmental conditions.

Results

Esculin is translocated with similar efficiency to sucrose

We previously described the specific phloem translocation of esculin, a naturally occurring fluorescent coumarin glucoside, by AtSUC2 and detailed how the glucoside moiety of esculin is required for recognition by the sucrose symporter (Knoblauch et al., 2015). We have also recently described the structural requirements of esculin for binding by AtSUC2 (De Moliner et al., 2018) Esculin does not enter the phloem in detectable amounts without AtSUC2 (Knoblauch et al, 2015; De Moliner et al., 2018). However, it was not known whether this probe was translocated as efficiently as sucrose. We tested this by comparing the translocation of esculin in intact seedlings over time with the translocation of ^{14}C labelled sucrose. Seedlings were tested at 7 days after germination (dag), and 0.3 µl of the adjuvant Adigor was added to each cotyledon shortly after dawn to facilitate loading through the cotyledons (Knoblauch et al., 2015). After 1 hour, either 0.3 µl of esculin or ^{14}C sucrose was loaded onto each cotyledon. The relative percentage of probe that was washed off the cotyledon, remained within the cotyledon, or had been translocated to the rest of the seedling was measured at 4 h post loading (Fig. 1) using either scintillation counting (^{14}C sucrose) or fluorescence readings calibrated against a standard curve (esculin). Whilst sucrose is converted into insoluble fractions, esculin enters vacuoles and is degraded over time (Knoblauch et al., 2015), making longer term comparisons between the two tracers unrealistic.

After 4 hours, the highest concentration of both ^{14}C-sucrose and esculin was present in the cotyledons (Fig. 1) and was similar for both probes (50%), as was the amount that remained on the surface of the cotyledon and that could be washed off. For both tracers, close to 20% of the total added to the leaf was translocated to the rest of the seedling by 4 h. This demonstrated that esculin was translocated in Arabidopsis seedlings as efficiently as sucrose.

Influence of environmental conditions on PTV

As the major solute carried within the phloem is sucrose, it has long been used as a proxy for describing the velocity of phloem transport. Based on the pressure-flow hypothesis, it is a

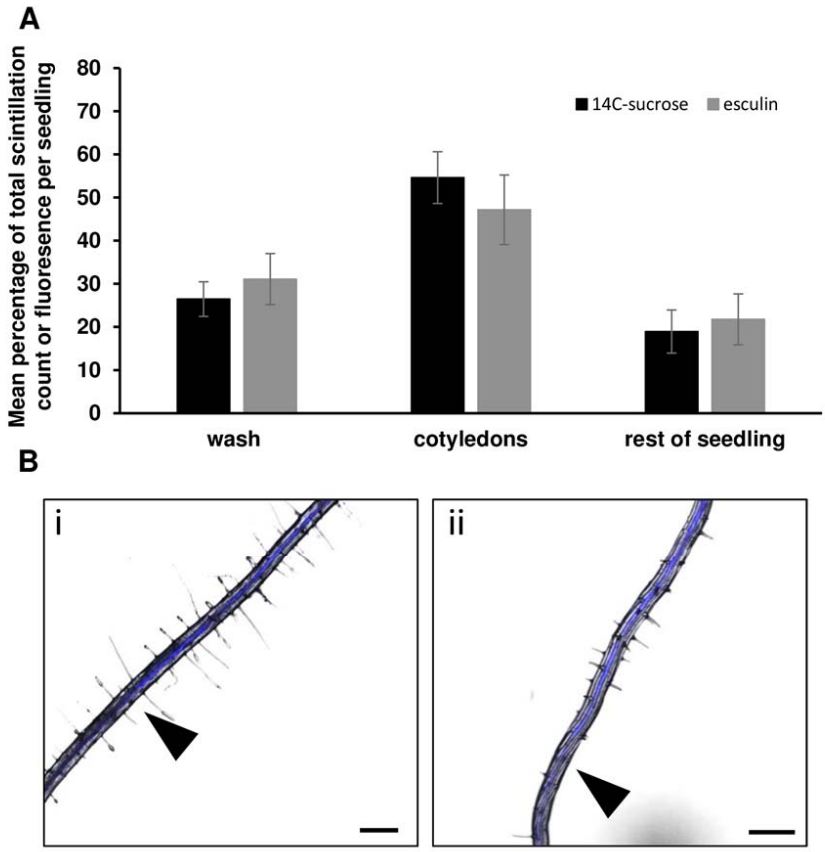

Figure 1. Comparison of the translocation of ^{14}C-sucrose and the phloem-mobile fluorescent probe esculin. (**A**) Black bars, ^{14}C-sucrose, grey bars, esculin. Mean percentage of total scintillation counts per seedling following application of ^{14}C-sucrose or mean percentage of total fluorescence per seedling following application of esculin. Both measurements taken 4 hours after application to cotyledons. Each bar represents a minimum of 10 seedlings across two independent experiments. Error bars = SEM (**B**) Esculin translocating in the root of a 7-day-old Arabidopsis seedling following application to the cotyledons i) early in the phloem, first point marked and time noted for velocity measurements ii) moving towards the root tip in the phloem, second point marked. Bar = 0.5 mm.

196 reasonable assumption that factors affecting the rate of photosynthesis, and thus the amounts
197 of sucrose produced, could affect the velocity of phloem transport. However, few studies
198 have been carried out on the direct impact of environmental changes on PTV. Those that
199 have been conducted used relatively mature plants and complex methods for measurement,
200 which reduce the opportunity for larger datasets and the testing of multiple experimental
201 parameters (Mullendore et al., 2010; Savage et al., 2013; Knoblauch et al., 2016).
202
203 Young Arabidopsis seedlings provide an uncomplicated model to study PTV. At 7 dag the
204 architecture is simple, consisting of two expanded cotyledons (source), a hypocotyl (path)
205 and a primary root tip (sink) that functions as the recipient of assimilates. Furthermore, the
206 phloem is arranged in two distinct poles, adjacent to the xylem poles, allowing for easy

identification and monitoring of flow in the translucent roots. Esculin was used to measure PTV in live seedlings. The probe was applied to both the cotyledons of seedlings as described above and the plants kept under standardized environmental conditions. The roots were then monitored with an epifluorescence microscope for the arrival of esculin in the phloem (Fig. 1B). The position of the fluorescent front was marked on the back of the plate and the seedlings were returned to the same growth conditions for a further 10 – 20 min. The seedlings were then re-imaged and the new esculin front was marked on the plate. Marks were checked for the accuracy of their position immediately after being made, and exact times were recorded. The basipetal phloem velocity in the root was then calculated as distance/time ($v = s/t$). This method measures only the visible front, and there may be undetectable levels of esculin ahead of this front. However, as the aim was to compare an estimate of PTV rapidly across large numbers of live seedlings, the accuracy is more than sufficient to allow these relative comparisons, rather than the absolute measurements provided by, for example, photobleaching methods (Jensen et al., 2011; Savage et al., 2013), and also avoids the effects of exposure to excess light.

Exogenous sucrose application to roots inhibits PTV

The pressure-flow hypothesis requires a pressure differential between photosynthetic source tissues and sink tissues, where the assimilates are unloaded and utilized for growth. Thus, increasing the source strength, by increasing sucrose availability, should in theory increase the pressure differential and increase PTV. This has been tested previously by feeding sucrose to excised leaves of several species (Vaughn et al., 2002; Lobo et al., 2015). The reverse should also be true, i.e., a reduction in sink strength by providing exogenous sucrose to the root, should lead to a reduction in PTV by decreasing sink strength. We tested this by growing seedlings on media containing either 0%, 1% (30 mM) or 2% (60 mM) sucrose, the latter two being standard concentrations used in growth media for Arabidopsis seedlings. Reducing the sink strength by supplying 30 mM sucrose to the root significantly reduced the PTV by more than 2-fold, as measured by esculin transport, compared to growth on media containing no sucrose (Fig. 2A). Growth on media containing 60 mM sucrose did not have a further effect, suggesting that the response to exogenous sucrose was saturated. Growth on the same concentrations of the non-metabolised osmolyte, mannitol, showed no effect at 30 mM and a slight increase in PTV at the higher concentration of 60 mM, demonstrating that the reduction in PTV is likely due to the exogenous sucrose, not changes in osmotic potential

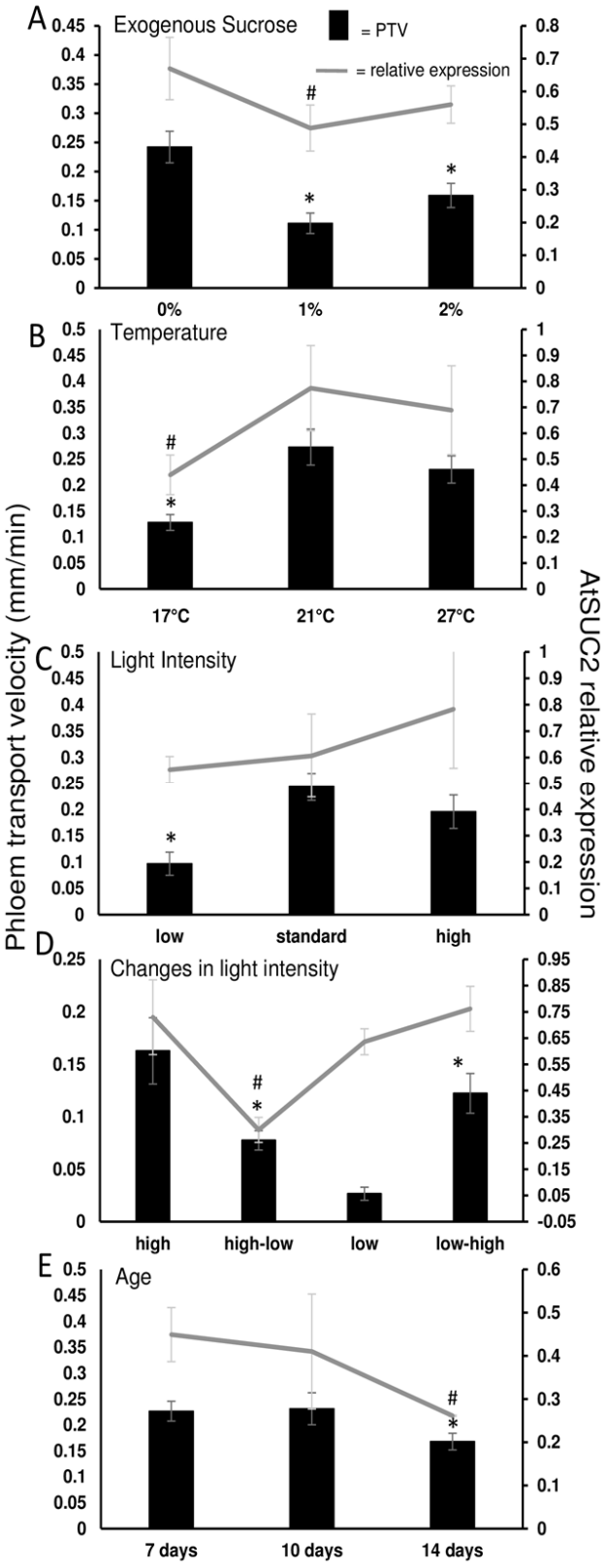

Figure 2. Variations in environmental conditions affect phloem velocity and partially regulate *AtSUC2* expression. (A) Exogenous sucrose concentration, (B) Growth Temperature, (C) Light Intensity, (D) Dynamic response to changes in light intensity, (E) Seedling Age (days after germination). Primary Y-axis is phloem transport velocity, error bars = SEM, n=minimum of 25 across minimum of 3 independent biological replicates. Secondary Y-axis is relative expression of *AtSUC2*. Error bars = SEM from 4 independent biological replicates. * or # indicates a p-value of ≤ 0.05 determined by t-test, for PTV or *AtSUC2* expression respectively, compared with the relevant control.

241 (Supplementary Figure S1). However, the PTV is not altered in seedlings transiently exposed

to increased sucrose concentrations for short periods (Supplementary Fig. S2).

Temperature affects PTV

Seedlings grown at low temperatures are generally smaller, and yet are often as photosynthetically active, as those grown at higher temperatures (Strand et al., 1999). Low temperatures have also been shown to induce adaptations for acclimation, including an increase in CC and SE numbers amongst the collection phloem of leaves (Cohu et al., 2013). When plants were exposed to cool temperatures, whilst light intensity remained at more optimal levels, sucrose accumulated in the leaves, suggesting that demand falls below production (Pollock, 1987). Short-term drops in the daytime temperature reduce the rate of photosynthesis (Pyl et al., 2012). This lowers sucrose production and results in a weaker source strength. To test the effect of weakening or strengthening the source via temperature we measured the PTV of plants grown at low and high temperatures (Fig. 2B). To ameliorate some of the potential effects of plant size on the resultant PTV, seedlings were grown for 4 days at 21^{o}C constant temperature and were then transferred to either 17^{o}C or 27^{o}C for 3 days. Seedlings grown at 17^{o}C showed more than a 2-fold reduction in PTV (0.13 ± 0.019 mm min^{-1}) compared to those at the standard temperature of 21^{o}C (0.28 ± 0.039 mm min^{-1}; Fig. 2B). Interestingly, there was no significant increase in the PTV of seedlings grown at 27^{o}C, despite a 2-fold increase in seedling biomass (Fig. 2B, S3).

Light intensity alters PTV

Generally, photosynthetic output increases with light intensity, until an optimum or capacity is reached. Photosynthetic capacity is significantly greater in leaves of apoplastic loading plants grown at high light compared with those grown under low light conditions (Amiard et al., 2005). This means that sucrose production is reduced under low light.

At low light levels, (< 10 µmol m^{-2} s^{-1}) the PTV in seedlings was 2-fold slower than in those grown under our standard light conditions (0.097 ± 0.022 vs 0.24 ± 0.026 mm min^{-1}), supporting the idea that a reduction in sucrose production, and thus source strength, reduces the overall pressure gradient in the phloem. Growth under high light resulted in a PTV similar to that under standard conditions (Fig. 2C).

Several apoplastic loaders undergo physical changes when switched from low light to high light conditions. Among these are alterations in plasma membrane surface area, which are thought to increase phloem loading capacity (Amiard et al., 2005). Such physical acclimation takes place over several days. We tested whether PTV could respond more dynamically to a short-term change in light intensity. Plants were grown under low, standard, or high light conditions (9, 100 and 190 μmol m^{-2} s^{-1}, respectively). At dawn, prior to measurement, the seedlings were transferred from either low light (LL) to high light (HL), or vice versa, for 2 h. The PTV displayed a clear response to the change in light intensities (Fig. 2D). There was a significant drop in PTV when plants were switched from HL to LL compared with those that remained at HL (0.078 ± 0.009 from 0.16 ± 0.032 mm min^{-1}). The reverse scenario, moving from LL to HL, increased the PTV from 0.027 ± 0.0062 to 0.12 ± 0.019 mm min^{-1}. This provides evidence that PTV responds, within relatively short time frames, to metabolic or photosynthetic changes that affect source strength.

PTV in seedlings of different ages

As seedlings develop, new leaves undergo the sink-source transition before they are fully expanded (Wright et al., 2003; Fitzgibbon et al., 2013). Once the first true leaves become carbon sources, the cotyledons are not required to produce sucrose to the same extent as before. We measured the PTV in seedlings grown for 7, 10 and 14 days after germination (dag) under our standard conditions (Fig. 2E). There was no difference in PTV between 7 and 10 dag, but a significant drop in PTV at 14 dag (0.23 ± 0.019 vs 0.17 ± 0.015). This decline may be due to decreased export from the cotyledons. However, by 14 dag a number of lateral roots have developed, potentially diluting the sink strength of the primary root tip. We attempted to discriminate between these confounding factors by comparing the PTV in 14 dag seedlings by loading esculin onto either the cotyledons or the true leaves (Supplementary Fig. S4). There was no significant difference in velocity between the two, although both were slower than the cotyledon-derived PTV seen at 7 or 10 dag, suggesting that the true leaves had indeed taken over some of the export duties.

***AtSUC2* expression is regulated in response to environmental cues**

AtSUC2 is expressed specifically in the CCs of the source phloem (Truernit and Sauer, 1994). The expression of *AtSUC2* has been shown previously to be regulated by leaf developmental stage and abiotic stresses (Truernit and Sauer, 1994; Gong et al., 2014; Durand et al., 2016). Such changes in expression were linked directly to sucrose levels in the sugar beet homologue, *BvSUT1* (Vaughn et al., 2002), where both the levels of BvSUT1 protein and carbon export were reduced in leaves supplied with exogenous sucrose. The tomato homologue, *LeSUT1* also shows transcriptional regulation by light (Kühn et al., 1997). Additionally, *Atsuc2-1* mutants are severely restricted in their growth and development but their phenotype can be partially rescued by growth on media supplemented with sucrose (Gottwald at al., 2000). As the primary role of AtSUC2 is to load sucrose into the CCs, it is a clear candidate for regulating PTV, although this has not been directly demonstrated. We therefore examined the expression of *AtSUC2* by RT-qPCR under the same environmental conditions that induced alterations in the PTV. To allow easy comparison with the PTV response, we plotted *AtSUC2* relative expression on a secondary Y-axis along with the PTV results for each environmental condition (Fig. 2).

Expression of *AtSUC2* was reduced in seedlings grown on media containing sucrose (Fig. 2A). Relative expression in seedlings grown without exogenous sucrose was 0.67 ± 0.095, reducing to 0.49 ± 0.07 in seedlings grown on 1% sucrose. There was no significant change in *AtSUC2* expression between plants grown on 1% and 2% sucrose, mirroring the results seen for PTV (Fig. 2A).

AtSUC2 expression levels increased with temperature, from 0.44 ± 0.08 in seedlings grown at 17^{o}C to 0.77 ± 0.16 at 21^{o}C and 0.69 ± 0.17 at 27^{o}C (Fig. 1B). This was a similar trend to the PTV results where the difference between 21^{o}C and 27^{o}C was also not significant (Fig. 2B).

The expression of *AtSUC2* under different light intensities was very variable across the biological replicates and, despite the mean levels following a general increase in expression from low light to high light, there was no significant difference between the light conditions (low light, 0.55 ± 0.05; standard light, 0.61 ± 0.16; high light, 0.78 ± 0.22; Fig. 2C). Interestingly, despite clear effects on the PTV, dynamic changes in light intensity only

produced a significant change in expression levels of *AtSUC2* following a change from HL to LL (Fig. 2D).

The expression of *AtSUC2* also altered with plant developmental stage (Fig. 1E). There was no significant difference between 7 and 10 dag, but by 14 dag the relative expression had decreased from 0.41 ± 0.13 to 0.26 ± 0.0015 (Fig. 1E).

PTV varies diurnally

Photosynthetic rate is inextricably linked to light perception and the circadian clock, and plants adapt their physiological and metabolic processes in order to optimize growth under different photoperiods (Sulpice et al., 2014). We investigated whether PTV varied diurnally, mirroring the differences seen in sucrose production under different photoperiods (Sulpice et al., 2014). Under long day conditions (LD; 16 hours light:8 hours dark) the PTV peaked around dawn (ZT0; Zeitgeber Time 0) at 0.21 (± 0.022) mm min^{-1} and gradually decreased over the day to its lowest rate of 0.11 mm min^{-1} (± 0.011) at ZT20 in the dark (Fig. 3A). A decrease in PTV from a daytime peak at ZT4 was also observed under short days (SD; 8 hours light:16 hours dark), but this plateaued in the dark from ZT16 onwards (Fig. 3B).

As the PTV varied diurnally, *AtSUC2* expression was tested for a circadian clock-linked expression profile (Fig. S5). The expression profile showed expression peaking in the dark period between ZT20 and ZT24 (Fig. S5A). However, the expression of *AtSUC2* was also strongly linked to the transitions between light and dark, suggesting that other factors may be involved in controlling the expression levels. In order to test whether the changes in expression were driven by light or the circadian clock, we tested the expression levels in seedlings entrained under the same LD period, but then grown for a further 2 days in constant light. The peaks of expression were reduced when the seedlings were switched to constant light, suggesting that there is a strong element of light regulation in the expression of *AtSUC2* (Fig. S5B).

Accumulation of sucrose in the cotyledons varies under differing environmental conditions

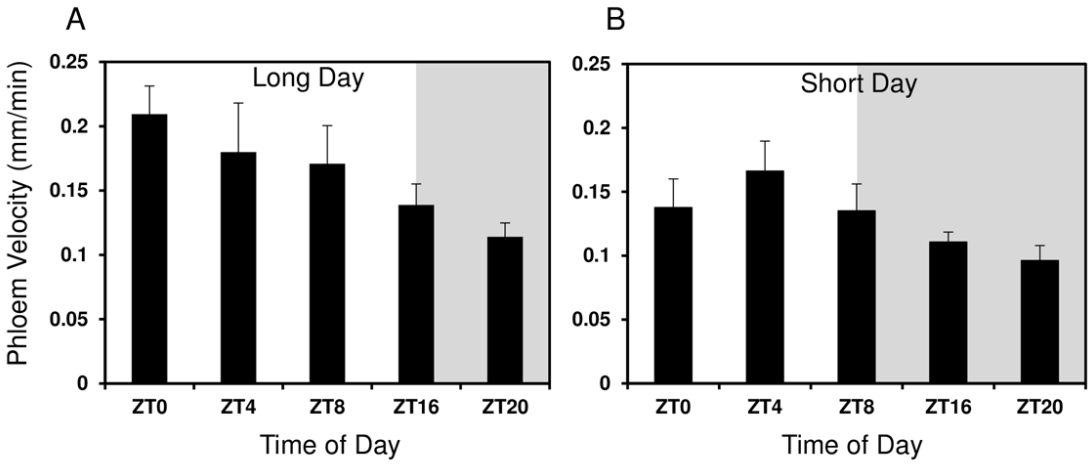

Figure 3. Phloem transport velocity varies throughout the day. (**A**) Under long day conditions (16 hours light:8 hours dark) and (**B**) short day conditions (8 hours light:16 hours dark). ZT0 = Dawn. Shaded areas represent the relevant period of dark. Error bars = SEM, n = minimum of 25 across minimum of 3 independent biological replicates.

To verify whether the control of PTV or the expression of *AtSUC2* was linked to changes in source and sink strength, we determined the amount of sucrose accumulated in the cotyledons and the amount present in the rest of the seedling under the range of environmental conditions used to examine PTV (Fig. 4A-F). At 17 ^{o}C, sucrose was present in the cotyledons at similar levels to seedlings grown under 21^{o}C. Seedlings grown under 27 ^{o}C had significantly less sucrose per gram fresh weight in their cotyledons (0.87 ± 0.32 vs 2.1 ± 0.37), yet similar amounts were present in the rest of the seedling (Fig. 4A). Of course, measurement of sucrose concentration in the rest of the seedling only provides a partial indication of the exported sucrose. It does not provide a complete measurement as it only accounts for sucrose that has not yet been metabolised or utilised for increases in biomass, the rate of which is also likely to vary under different environmental conditions.

Under low light conditions, very little sucrose was present in the cotyledons, although similar amounts were present in the rest of the seedlings compared with those grown under standard light (Fig. 4B). Under high light more sucrose was present than under low light, but not significantly more than under standard light intensity in the rest of the seedling (Fig. 4B).

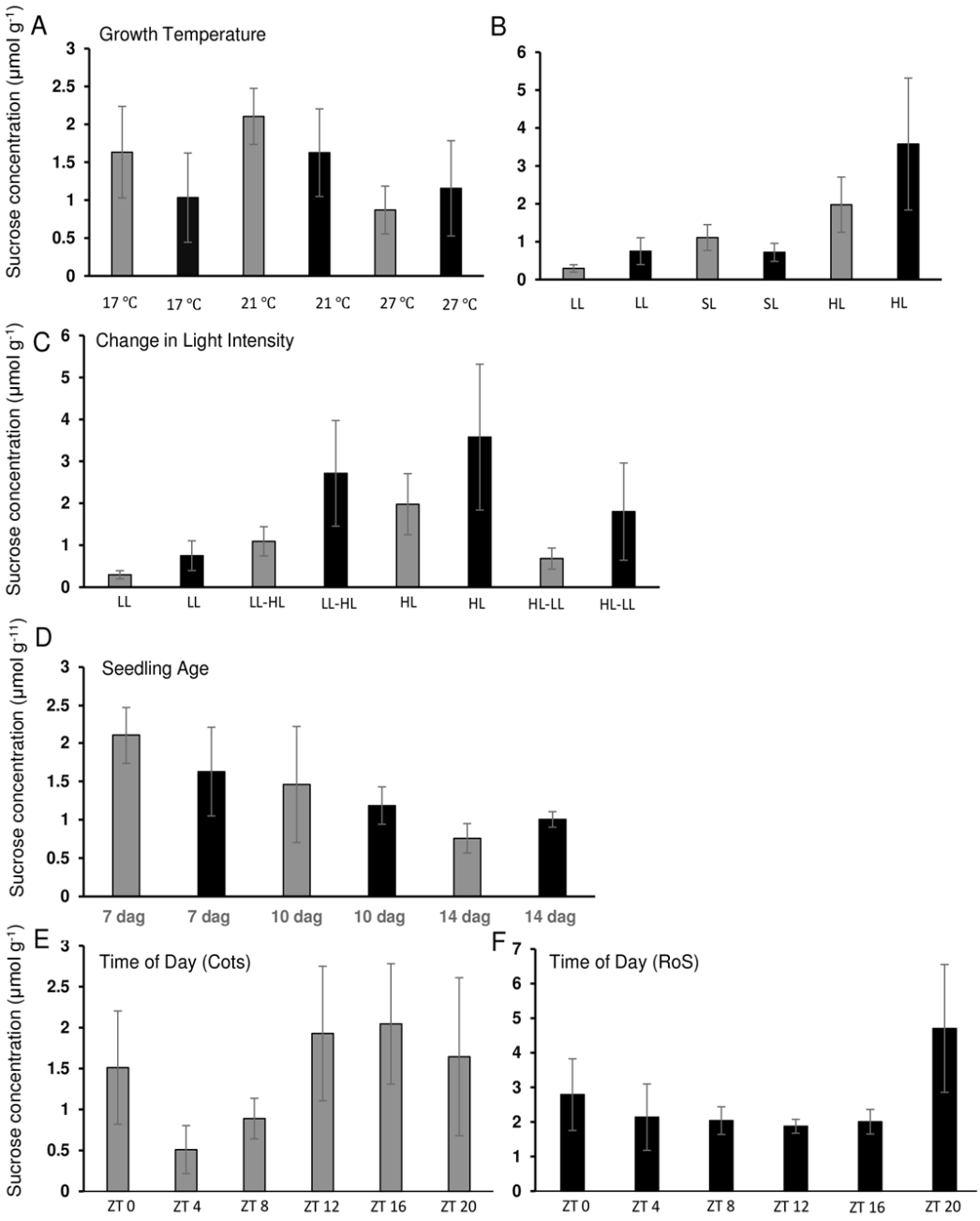

Figure 4. Sucrose concentration in both the source and sink tissues varies under different environmental conditions. Sucrose concentration was measured in an enzymatic assay from the cotyledons (Cots, grey bars) and the rest of the seedling (RoS, black bars) grown under a range of environmental conditions. (A) Growth temperature, (B) Light intensity, LL= Low Light, SL= Standard Light, HL=High Light, (C) Dynamic changes in light intensity, seedlings grown under one light intensity were switched to the opposite at dawn and harvested after 2 hours, (D) Seedling age, (E) Time of Day, cotyledons only. ZT = Zeitgeber Time, ZT0 is defined as time of lights on. (F) Time of Day, RoS only. Error bars represent SEM, n= 4 across 3 independent replicates. * indicates a p-value of ≤ 0.05 for sucrose concentration compared with the relevant control calculated by t-test on log-transformed data.

389 This suggests that lower light intensity results in lower rates of photosynthesis, potentially
390 resulting in lower concentrations of sucrose exported. After just 2 hours in high light,

seedlings previously grown at low light showed an increase in the mean amount of sucrose, compared with those maintained at low light (1.09 ± 0.35 vs 0.3 ± 0.1), and much of this had already been exported to the rest of the seedling (Fig. 4C), at levels comparable with seedlings grown under high light. The opposite was also true. Seedlings grown at high light and transferred to low light for 2 hours at dawn displayed lower levels of sucrose in their cotyledons than those at continuous high light, although still higher than those grown continuously under low light (Fig. 4C; 0.68 ± 0.25 vs 0.3 ± 0.1).

Seedling age had a significant impact on the concentration of available sucrose (Fig. 4D), with a reduction in concentration found in the cotyledons at 14 dag, suggesting that the first leaves are beginning to take over the role of carbon sources. There were some differences in sucrose concentration across the day in the cotyledons, with the peak concentration occurring towards the end of the day at ZT12 and during the night and reducing through the night to the lowest levels by early morning (ZT4) (Fig. 4E & F).

PTV remains sensitive to environmental changes when *AtSUC2* is expressed from an exotic promoter

409 Sucrose has been shown to negatively regulate *AtSUC2* expression (Dasgupta, et al., 2014),
410 however our results were not always conclusive regarding *AtSUC2* expression level and the
411 corresponding PTV (Fig. 2). When *AtSUC2* is expressed from a CC-localized promoter from
412 *Commelina Yellow Mottle Virus* (CoYMV), it rescues the phenotype of *atsuc2* knockout (ko)
413 plants, indicating that it works as a functional replacement for native *AtSUC2* (Srivastava et
414 al., 2009). Therefore, we used *CoYMV:AtSUC2* lines to examine whether the regulation of

PTV via *AtSUC2* required the *AtSUC2* promoter. Under each set of environmental conditions, *CoYMV:AtSUC2* seedlings behaved similarly to *AtSUC2:AtSUC2* lines, suggesting that, despite the effects seen on the abundance of *AtSUC2* transcripts, PTV regulation does not depend entirely on transcriptional regulation from the *AtSUC2* promoter (Fig. 5 A-D). However, some minor differences were noted, in particular the PTV did not respond to low light nor acute changes in light as dynamically in *CoYMV:AtSUC2* seedlings

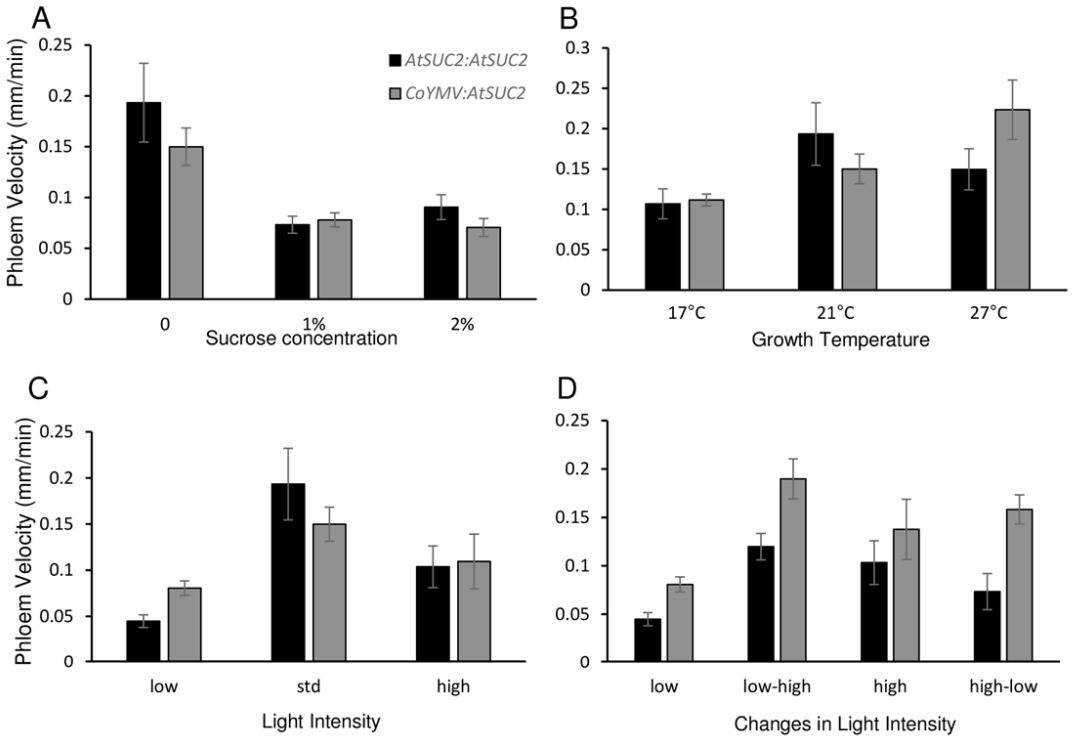

Figure 5. Phloem velocity remains responsive to environmental conditions when *AtSUC2* is expressed under a phloem-specific foreign promoter. Esculin translocation was used to measure phloem velocity under a range of environmental conditions in 7-day-old *atsuc2-1* seedlings expressing either *AtSUC2pro:AtSUC2* or *CoYMVpro:AtSUC2*. (**A**) Exogenous sucrose concentration, (**B**) Growth Temperature, (**C**) Light Intensity, (**D**) Dynamic response to changes in light intensity. Error bars represent SEM, n=minimum of 10 seedlings across 3 replicates.

compared to *AtSUC2:AtSUC2* (Fig. 5D; LL = 0.045 ± 0.0068 vs 0.081 ± 0.0078, LL-HL = 0.12 ± 0.014 vs 0.19 ± 0.021), or as described previously for wild type (Fig. 2D). This suggests that the *AtSUC2* promoter may be required to regulate the PTV under acute changes in light intensity.

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Discussion

Esculin facilitates high-throughput measurement of PTV in young seedlings

The fluorescence of esculin and its recognition by AtSUC2, provides a straightforward mechanism for monitoring phloem transport *in planta*. This method allows relatively high-throughput studies to be conducted in live seedlings, where growth on agar plates in environmentally controlled growth chambers allows perturbation of environmental conditions and measurement of the effects of such changes on PTV. We have shown that esculin is an excellent proxy for sucrose in this type of study, as it is translocated from the cotyledon surface to the rest of the seedling with the same efficiency as radiolabelled sucrose (Fig. 1). Furthermore, esculin only enters the phloem in Arabidopsis in detectable amounts using the AtSUC2 symporter, the key sucrose loader in Arabidopsis (Knoblauch et al, 2015; De Moliner et al, 2018).

PTV has been measured in a number of different species but previous studies focused on large, mature plants due to the technical limitations of the available methods. The reported PTV, measured by MRI or fluorescent tracer methods, varies across species, tissues and developmental stages, ranging from 0.18 mm min^{-1} to 102 mm min^{-1}. The majority of species that have been measured show a PTV of around 15 mm min^{-1} (Windt et al., 2006; Jensen et al., 2011). The results presented here indicate that the velocity (around 0.25 mm min^{-1}) in young Arabidopsis seedling roots, measured under normal growth conditions, is towards the slower end of this scale. A velocity of 1.5 mm min^{-1} was reported for individual cells in the region where the metaphloem transfers assimilates to the protophloem, slowing to 0.3 mm min^{-1} in cells in the protophloem unloading zone (Ross-Elliott et al., 2017). Our measurements were made in roots before esculin had reached the unloading zone, but ultimately represent an average of the cells within the measured root region, rather than the velocity within a single sieve element. Although measuring PTV at the single-cell level is possible with esculin, it is much more time-consuming, and thus does not lend itself to the high-throughput method described here. Our method allowed us to rapidly monitor the effects of environmental variation on the PTV, and could be easily adapted for use in older plants and other species. The simplicity of this method does not account for esculin moving ahead of the visible front, nor does it compensate for any lateral loss of esculin along the phloem

pathway, thus the relative PTV measurements presented here are likely to be slower than the absolute PTV for sucrose. Further, as any lateral loss from the phloem is undetected, we cannot determine whether any environmental variables induce changes in the rate of lateral loss, which would contribute to the effect seen on the PTV. However, our method is a significant advantage over the use of radiolabelled sucrose, which does not permit the level of resolution we have reported here.

Environmental conditions affect PTV

Whilst it has generally been assumed that environmental conditions, particularly those likely to affect the production or metabolism of sucrose, could have a substantial effect on the PTV, actual data has been limited to a relatively small number of studies, one of which showed that developmental stage in cucurbits affected PTV (Savage et al., 2013) and another which detailed the effects of developmental stage and osmotic stress in Arabidopsis (Durand et al., 2017). Other studies suggest that, despite fluctuations in carbon export over the day and night, PTV remains more or less constant in the stems of several species (Windt et al., 2006). These authors argued that the PTV was likely to be regulated to a consistent velocity in order to allow constant transmission of long-distance molecular signals through the phloem. Our results contrast with this, showing that PTV in Arabidopsis varies markedly in response to a variety of stimuli.

Growth at low temperatures, low light and acute changes from high to low light, resulted in significant reductions in PTV (Fig. 2). Furthermore, in contrast to the results obtained for 3 out of 4 species tested by Windt et al (2006), there was a distinct diurnal variation in PTV in Arabidopsis, with the lowest velocity recorded late in the night (Fig. 3). These are all environmental conditions that affect the rate of photosynthesis and thus sucrose production. This indicates that PTV may be related to the amount of sucrose available for export.

Is PTV regulated by complex signals or simply by source/sink strength?

The Münch hypothesis states that the phloem flow is created by the disparity between the concentration of sucrose in the source and sink tissues. The stronger the demand for carbon in the sink tissues, the more sucrose is required to be loaded into the collection phloem in the

source tissues. However, what happens if the sink strength is reduced? We tested this using exogenously applied sucrose to the roots. This resulted in PTV being reduced by around half (Fig. 2).

Many researchers grow Arabidopsis seedlings on media supplemented with sucrose, despite the fact that sucrose can change the expression of more than 797 genes and regulates many of the elements of the circadian clock (Gonzali et al., 2006; Dalchau et al., 2011). Unfortunately, much of the experimental data publicly available has been produced using seedlings grown on sucrose. Clearly, care must be taken when interpreting such data, especially expression data for sucrose-related genes.

It has been shown that a reduction in sink strength leads to sucrose accumulation in source leaves, which in turn inhibits photosynthesis, reducing the sucrose available for transport (Paul and Pellny, 2003). Therefore, conditions that naturally reduce the efficiency of photosynthesis should also reduce the amount of sucrose available for transport. Our results corroborate this model, as under low light (< 10 µmol m^{-2} s^{-1}) PTV is reduced and so is the sucrose concentration (Fig. 2C-D & Fig. 4 B & C). Equally, as the seedling develops, the cotyledons begin to senesce and therefore become less important as the first true leaves take on a more dominant role in carbon export. In our experiments this could be seen as a drop in PTV in seedlings at 14 dag (Fig. 2E), attributed at least in part to reduced export from the cotyledons and increased export from the true leaves (Supplementary Fig. S4), coupled to a corresponding reduction in sucrose levels in the cotyledons (Fig. 4D). However, it has been postulated that decreased PTV, caused by reduced sink strength, would not be sufficient to alter the concentration of sucrose in the mesophyll in active apoplastic loaders such as Arabidopsis, as the proton motive force that drives the sucrose symporters enables phloem loading at even very high apoplastic sucrose concentrations (Ainsworth and Bush, 2011). Further, there is some evidence to suggest that K+ ions have a role in maintaining the hydraulic pressure gradient of the phloem pathway, playing a role in reloading leaked sucrose (Deeken et al 2002). Therefore, this would suggest that phloem loading must be down-regulated to allow sucrose to build up in the mesophyll cells. We asked whether this response could occur through a feedback signal on the transcription rates of the *AtSUC2* gene, thus reducing the number of transporters available. This would provide a natural ‘brake’ on the amount of sucrose being loaded into the phloem, reducing flow. Indeed, there was a slight

reduction in the expression of the main sucrose transporter, *AtSUC2,* when seedlings were grown on exogenous sucrose, suggesting that the PTV may, at least in part, be linked to the transcriptional regulation of the transporter. A homologue of *AtSUC2*, the sugar beet *BvSUT1,* is transcriptionally repressed by exogenous sucrose applied to leaf discs (Vaughn et al., 2002), and sucrose transport activity and mRNA abundance were decreased in leaves fed exogenous sucrose via the xylem (Chiou and Bush, 1998). This is not simply an osmotic stress effect, as seedlings grown on mannitol did not show a decrease in PTV (Fig S1 and S2). In fact, at 60 mM, mannitol induced an increase in PTV. Such opposing effects have been previously reported in pea seedlings, although the increase was triggered at even lower concentrations of mannitol (Schulz, 1994). Schulz (1994) suggests that the increase in PTV following mannitol treatments serves to counteract the effects of osmotic stress where higher amounts of solutes are drawn from the phloem in response to the low apoplasmic potential. However, in pea seedlings, as in our Arabidopsis seedlings, low concentrations of sucrose (< 75 mM) result in an inhibition of phloem transport. One potential explanation for this is that the uptake of sucrose into the sink cells would reduce the exosmosis of water (Schulz, 1994). Such effects are usually associated with short-term osmotic stress, with equilibrium being reached in the root tip after a few hours (Schulz, 1994), yet our seedlings were grown on media from germination. Interestingly, the observed effects may not be solely an osmotic response, as sucrose and mannitol have previously been shown to have opposite effects on the activity of the *AtSUC2* promoter, (Dasgupta et al, 2014). Equally, it is not possible to rule out from these experiments that the sucrose effect on PTV is not partially caused by physical changes in the phloem and a general difference in biomass caused by growth on exogenous sucrose (Fig S3). When seedlings were transferred from media without any sucrose or mannitol to media containing either 30 mM or 60 mM of either sucrose or mannitol for 2 hours, no reduction in PTV was seen, although the increase in PTV caused by a high concentration mannitol was replicated (Fig S2). This may mean that the sucrose effect on PTV does require structural changes to the phloem or, as the reduction in import from the phloem was seen following similarly short timescales in pea seedlings, it may suggest that 2 hours is not sufficient for the uptake into the roots from solid media, as opposed to root tips submerged in liquid media (Schulz, 1994). Equally, the timescale may be too short for a potential feedback signal from sink to source to have a significant effect on the PTV.

Not all environmental conditions that affected PTV and sucrose accumulation caused a significant change in the expression levels of *AtSUC2* (Fig. 1). Seedlings grown under low

565 temperatures showed a decrease in *AtSUC2* expression but seedlings grown under low light,
566 despite a significant change in PTV, did not (Fig. 1C). Intriguingly, a significant drop in
567 expression was seen for seedlings grown under high light and then moved to low light for 2
568 hours. This indicates that perhaps the direct control of expression is used to deal with
569 dynamic short-term fluctuations in the environment.
570
571 Under consistent low light, there was essentially no change in *AtSUC2* expression despite a
572 significant reduction in PTV. Xu et al. (2018) found that Arabidopsis and other apoplastic
573 loaders, grown under low light conditions, did show reduced expression of a range of sucrose
574 transporters (Xu et al, 2018). However, their low light conditions were at 40 µmol m^{-2} s^{-1},
575 whereas in the current study they were significantly lower (10 µmol m^{-2} s^{-1}). We did see a
576 significant reduction of *AtSUC2* expression in plants moved from high light to low light for 2
577 hours, supporting the idea that there is dynamic regulation of the promoter under acute
578 environmental changes. Further, we cannot rule out that there may be morphological
579 alterations induced in the root phloem in seedlings grown under low-light or low-temperature
580 conditions, which may well affect the PTV, although they were only grown under such
581 conditions for 3 days in order to try to reduce such effects.
582
583 By expressing AtSUC2 in an *atsuc2ko* background, using a phloem specific promoter from
584 *Commelina Yellow Mottle Virus* (*CoYMV*), we were able to test whether the dynamic changes
585 seen in PTV were the result of promoter-specific changes in *AtSUC2* expression (Srivastava
586 et al., 2009). Despite previous work showing that exogenous sucrose inhibits promoter
587 activity, and our RT-qPCR data showing mild effects on the expression levels of *AtSUC2*
588 under certain conditions, this was not the case; PTV responded to the different environmental
589 cues in a similar way in both *CoYMV:AtSUC2* seedlings and *AtSUC2:AtSUC2* seedlings (Fig.
590 5). The main exceptions involved low light and acute changes in light intensity. Here, the
591 *CoYMV:AtSUC2* seedlings were less responsive to a change from high to low light,
592 suggesting that under these conditions transcriptional repression of *AtSUC2* from the
593 promoter may indeed play a more important role.
594
595 It is likely that several factors converge on SUTs to fine-tune their transcriptional regulation.
596 For example, blocking protein phosphatase activity in sugar beet resulted in a decrease in
597 symporter transcript abundance, and ultimately symporter abundance (Ransom-Hodgkins et

al., 2003). Further experiments suggested that there might be a phosphorylated protein that is a negative regulator of BvSUt1 transcription (Ransom-Hodgkins et al., 2003). Other factors have been shown to be involved in regulation of SUTs. For example, StSUT4 accumulates under far-red light conditions (Liesche et al., 2011). However, it also accumulates following actinomycin D treatment, suggesting the accumulation is due to increased transcript stability, not increased transcription (Liesche et al., 2011). Indeed, further regulation is likely to occur at the post-transcriptional level as most SUT mRNAs are relatively short-lived, with half-lives ranging from 60 – 130 minutes (Vaughn et al., 2002; He et al., 2008: Liesche et al., 2011)

Control of transporter activity clearly also occurs at the post-translational level, with many SUTs proving to be relatively unstable proteins; StSUT1 is degraded in < 4 hours and BvSUT1 in just 2.7 hours (Vaughn et al., 2002). Xu et al. reported an increase in LeSUT1 abundance under high light conditions, despite a lack of transcriptional change, suggesting that there is either post-transcriptional or translational regulation, such as an increase in protein stability (Xu et al., 2018). Some SUTs have also been shown to dimerise with different proteins. For SUT1 this is likely to be redox-dependent, with oxidizing conditions favouring homodimerisation and increased plasma membrane targeting (Reinders et al., 2002; Krügel et al., 2008). SUT4 has been suggested to act as an inhibitor of SUT1, thus inhibiting sucrose transport directly (Liesche et al., 2011). It is possible that a similar situation could occur in Arabidopsis. Another potential layer of regulation could come simply from the availability of protons required for the active loading of sucrose (Khadilkar et al., 2016).

Conclusion

Our data, using esculin as a proxy for sucrose, suggest that the expression, activity and stability of SUTs is dynamically regulated by a number of pathways. This fits with a physiological system that needs to be able to respond rapidly to sudden changes in environmental conditions that affect sucrose production and thus source-sink relations.

Materials and Methods

Plant growth

Seeds of Arabidopsis (*Arabidopsis thaliana*), ecotype Col-0 were surface sterilized by immersion in 10% v/v bleach for 15 minutes, then rinsed in 70% v/v ethanol, followed by 5 rinses in sterile ddH_2O. Seeds were plated in two rows, with an average of 15 seeds per plate on 25 ml 0.5 x MS basal salt media (Duchefa #MO221), solidified with 2% w/v Phytoagar (Melford # P1003). Seeds were stratified at 4 ^{o}C for two days before transfer to controlled environment growth chambers (Percival) within a climate controlled dark room. Standard conditions were 80-100 µmol m^{-2} s^{-1} white light, under long days (16 h light:8 h dark) at a constant 21 ^{o}C. Seeds of *atscu2-4 AtSUC2:AtSUC2* and *CoYMV:AtSUC2* were the kind gift of Brian Ayre and have been previously described (Srivastava et al., 2009)

Phloem transport efficiency of fluorescent probes vs radiolabelled sucrose

Seedlings were treated at 7 dag. Both cotyledons were pre-treated with 0.3 µl of a 2.5% v/v Adigor (Syngenta) solution for 1 hour. Then either 0.3 µl of 9 mg ml^{-1} Esculin, CTER or ^{14}C sucrose (Perkin-Elmer) was added to each cotyledon. Seedlings were sampled at 4 hours post probe application. Remnants of the probe solution were washed off by submerging the intact seedling's cotyledons into 600 µl of ethanol. The cotyledons were then removed into a separate tube of 600 µl ethanol and the remainder of the seedling (root, hypocotyl, meristem and emerging true leaves) into a third tube of 600 µl ethanol. All tubes were heated to 75 ^{o}C for 1 hour, chilled on ice briefly and then centrifuged at full speed for 2 minutes. For measurement, 300 µl of the radiolabelled samples was added to 3 ml of scintillant in a scintillation vial and counted on for 2 minutes per sample with two repeats. Fluorescent samples were split into 200 µl portions and loaded into separate wells in a 96-well plate (Greiner) before being read on a Tecan M200 with excitation set at 405 nm and emission collected at 454 nm for esculin. Control samples from seedlings not treated with esculin were used to give background readings and subtracted from all samples. A minimum of 5 seedlings per treatment and time-point were used, with two independent replicate experiments for each probe.

Measurement of phloem transport velocity

Except where noted, seedlings were pre-treated at ZT0/ putative dawn (lights-on) with 0.3 µl of 2.5% Adigor in ddH_2O for 1 hour. The cotyledons were blotted lightly to remove excess Adigor solution and then 0.3 µl of esculin was added to each cotyledon. After 10 minutes the seedlings were checked for the appearance of esculin in the phloem in the root. The fluorescent front was marked on the plate and time noted. Seedlings were rechecked and the new front marked together with time. The distance moved was calculated by measuring the root length between the two marks using ImageJ software. The velocity was calculated as: velocity = distance/time. A minimum of 25 seedlings, spread over a minimum of 3 independent replicates was used for each condition. In order to minimize differences caused by variation in growth and biomass, seedlings for temperature and light intensity experiments were grown under standard conditions for 4 days, before being transferred to the appropriate condition. For acute change in light intensity, seedlings were transferred to the opposite light intensity at ZT0/ putative dawn.

Gene Expression Measurements

Seedlings were grown as described above and 100 mg seedlings harvested per sample in duplicate, 1 hour after dawn or two hours following acute changes in light intensity. Time of day and circadian experiment seedlings were harvested at the time points described. Seedlings were harvested in 1 ml of RNAlater (Sigma) and kept in the dark overnight at 4 ^{o}C before being transferred to -20 ^{o}C until extraction. RNA was extracted using RNEasy Plant Mini Kit (Qiagen). Briefly, tissue was ground in 450 µl RLT buffer with two steel beads using a Tissuelyser Mixer Mill (Qiagen) for 2 min at 27 s^{-1}, the blocks were rotated and run again for 2 minutes. Samples were then incubated at 56 ^{o}C for 3 minutes before being added to the shredder columns and manufacturer's protocol was followed, including the DNAseI step. Samples were double-eluted in 35 µl of RNase free water. One µg RNA per sample was reverse-transcribed using a RevertAid First Strand cDNA Synthesis kit (Thermo Scientific). RT-qPCRs were set up using Roche Lightcyler 480 SYBR Green 1 Master mix in technical triplicates and cycled in a LightCycler 480. The mRNA abundance was calculated using the Relative Quantification function. Oligos used were AtSUC2 F-

GCAGACGGGTGAGTTAGA and AtSUC2 R-GGAGATTGGACCACAGAG (Durand et al., 2016) and for the reference gene,
ACT7 F –TGAACAATCGATGCACCTGA and ACT7 R-CAGTGTCTGGATCGGAGGAT.

Extraction and enzymatic quantification of sucrose

Seedlings were harvested, cotyledons and the rest of the seedling tissue were split into separate tubes, 1 ml of 70 % ethanol was added and then flash frozen in liquid N_2. For extraction, samples were boiled at 90 ^{o}C for 10 min, then spun down and supernatant transferred to a fresh Eppendorf. Then 250 µl of ddH_2O was added and samples vortexed for 1 min, before adding 250 µl ethanol and then boiled at 90 ^{o}C again for 10 minutes. Samples were then spun down and supernatant transferred to a new tube. The total volume was brought to 1.6 ml with ddH_2O and stored at -20 ^{o}C until quantification. In clear 96-well microtitre plates 200 µl of assay cocktail (10 mg NADP, 33 mg ATP dissolved in 40 ml of 150 mM Tris and 5 mM $MgCl_2$ (pH 8.1)) was added to each well, together with 45 µl ddH_2O and 5 µl of sample. Each sample was loaded in triplicate and mixed well. Absorbance at A_{340} (read A) was measured on a Fluostar Omega plate reader (BMG Labtech) and then 5 µl (0.5U) of Hexokinase and glucose-6-phosphate dehydrogenase was added to each well, mixed thoroughly and incubated at room temperature for 30 minutes. Then read at A_{340} again (read B). The initial reading (A) was subtracted from the second (B) to give the glucose level. In a second plate, 40 µl of 100 mM tri-sodium citrate with 5 mM $MgCl_2$ (pH 5) was added to each well, along with 5 µl of each sample and 4 µl of invertase and then incubated at room temperature for 10 minutes. Then 200 µl of assay cocktail was added, mixed and then the wells were read at A_{340} (read C), before adding 5 µl (0.5 U) of hexokinase/G6-PDH to each well and incubated at room temperature for 30 minutes before a final reading at A_{340} (read D). Sucrose dA_{340} = (D-C) – (B-A). Absorbency of NADPH is 6.22 so sucrose concentration (µmol) was calculated as $dA_{340}/6.22$ and then multiplied by 50 to account for the initial dilution. Each result was then divided by the fresh weight of the initial sample to give the concentrating in µmol g^{-1}.

Accession numbers

Sequence data for AtSUC2 can be found using accession number At1g22710.

730

731 **Supplementary Data**

732

733 The following supplemental materials are available:

734

735 **Supplementary Figure S1** Comparison of the effects of growth on different concentrations
736 of sucrose and mannitol on PTV.
737 **Supplementary Figure S2** Comparison of the effects of transient exposure to sucrose or
738 mannitol on PTV.
739 **Supplementary Figure S3**
740 Mean wet biomass for seedlings grown under a range of environmental conditions.
741 **Supplementary Figure S4**
742 Comparison of phloem velocity in 14-day-old seedlings following loading of cotyledons or
743 true leaves.
744 **Supplementary Figure S5**
745 *AtSUC2* expression is not tightly circadian but regulated by light signals.
746 **Supplementary Figure S6**
747 Root length of 7-day-old *AtSUC2:AtSUC2* and *CoYMV:AtSUC2* seedlings.

748

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750

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755

756 **Figure Legends**

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758

759 **Figure 1. Comparison of the translocation of ^{14}C-sucrose and the phloem-mobile**
760 **fluorescent probe esculin.** (**A**) Black bars, ^{14}C-sucrose, grey bars, esculin. Mean percentage
761 of total scintillation counts per seedling following application of ^{14}C-sucrose or mean
762 percentage of total fluorescence per seedling following application of esculin. Both
763 measurements taken 4 hours after application to cotyledons. Each bar represents a minimum

of 10 seedlings across two independent experiments. Error bars = SEM (**B**) Esculin translocating in the root of a 7-day-old Arabidopsis seedling following application to the cotyledons i) early in the phloem, first point marked and time noted for velocity measurements ii) moving towards the root tip in the phloem, second point marked. Bar = 0.5 mm.

Figure 2. Variations in environmental conditions affect phloem velocity and partially regulate *AtSUC2* expression. (A) Exogenous sucrose concentration, (B) Growth Temperature, (C) Light Intensity, (D) Dynamic response to changes in light intensity, (E) Seedling Age (days after germination). Primary Y-axis is phloem transport velocity, error bars = SEM, n=minimum of 25 across minimum of 3 independent biological replicates. Secondary Y-axis is relative expression of *AtSUC2*. Error bars = SEM from 4 independent biological replicates. * or # indicates a p-value of ≤ 0.05 determined by t-test, for PTV or *AtSUC2* expression respectively, compared with the relevant control.

Figure 3. Phloem transport velocity varies throughout the day. (**A**) Under long day conditions (16 hours light:8 hours dark) and (**B**) short day conditions (8 hours light:16 hours dark). ZT0 = Dawn. Shaded areas represent the relevant period of dark. Error bars = SEM, n=minimum of 25 seedlings across a minimum of 3 independent biological replicates

Figure 4. Sucrose concentration in both the source and sink tissues varies under different environmental conditions. Sucrose concentration was measured in an enzymatic assay from the cotyledons (Cots, grey bars) and the rest of the seedling (RoS, black bars) grown under a range of environmental conditions. (A) Growth temperature, (B) Light intensity, LL= Low Light, SL= Standard Light, HL=High Light, (C) Dynamic changes in light intensity, seedlings grown under one light intensity were switched to the opposite at dawn and harvested after 2 hours, (D) Seedling age, (E) Time of Day, cotyledons only. ZT = Zeitgeber Time, ZT0 is defined as time of lights on. (F) Time of Day, RoS only. Error bars represent SEM, n= 4 across 3 independent replicates. * indicates a p-value of ≤ 0.05 for sucrose concentration compared with the relevant control calculated by t-test on log-transformed data.

Figure 5. Phloem velocity remains responsive to environmental conditions when *AtSUC2* is expressed under a phloem-specific foreign promoter. Esculin translocation was used to measure phloem velocity under a range of environmental conditions in 7-day-old *atsuc2-1* seedlings expressing either *AtSUC2pro:AtSUC2* or *CoYMVpro:AtSUC2*. (**A**) Exogenous sucrose concentration, (**B**) Growth Temperature, (**C**) Light Intensity, (**D**) Dynamic response to changes in light intensity. Error bars represent SEM, n=minimum of 10 seedlings across 3 replicates.

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